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A genome scan of 18 families with chronic lymphocytic leukaemia

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Summary. Chronic lymphocytic leukaemia (CLL) accounts for about 30% of all leukaemias and is most prevalent in older individuals. Significant familial aggregation has been demonstrated but the mode of inheritance is unknown. Recurrent cytogenetic abnormalities are frequently found in CLL tumour cells but no susceptibility genes have been confirmed. We have collected clinical data and biospecimens on families ascertained for having at least two living patients with CLL. The current study included DNA samples from 94 individuals (38 affected patients) in 18 families. We have carried out a genome scan using the ABI 28-panel medium density linkage mapping set (average spacing of 10 cM and average heterozygosity of 80%). Genotypes for 359 markers were scored. Multipoint limit of detection (lod) scores were calculated, assuming both dominant and recessive inheritance and allowing for increased penetrance with age and genetic heterogeneity. Non-parametric linkage scores were also calculated. Lod scores of 1·0 or greater were found on regions of chromosomes 1, 3, 6, 12, 13 and 17, but none of these loci achieved statistical significance. Four of these six regions (6q, 13q, 12 and 17p) coincide with areas where cytogenetic abnormalities are frequently observed in CLL tumour cells and are, therefore, strong candidate regions for containing germ line changes.

Keywords: chronic lymphocytic leukaemia, genetics, family studies, linkage mapping, candidate genes.

B-cell chronic lymphocytic leukaemia (B-CLL) is a neoplastic disease characterized by the accumulation of small, mature-appearing lymphocytes in the blood, bone marrow and lymphoid tissues. It is the most common leukaemia among older adults in Western countries, accounting for about 30% of all leukaemia patients. Data from the United States Surveillance, Epidemiology and End Results Registry (SEER) estimate the US incidence to be 3·7/100 000 with a median age at diagnosis of 72 years (Ries *et al,* 2003). Incidence rates in men is nearly twice that in women. Although advanced age, Caucasian ancestry and family history of haematological malignancies are recognized risk factors, the aetiology of CLL is unknown. Case–control studies have evaluated diverse environmental and occupation exposures such as radiation, magnetic fields, viruses and pesticides, but associations with these exposures have been weak and inconsistent. Family history of CLL or other haematolymphoproliferative disorders is consistently found to be a risk factor for CLL (Cartwright *et al*, 1987; Linet *et al*, 1989; Radovanovic *et al*, 1994; Capalbo *et al,* 2000). A highly significant familial aggregation of CLL was shown among cancer subtypes in the Utah Cancer Registry (Cannon-Albright *et al*, 1994). Clinical descriptions of CLL families can be found in the literature (Yuille *et al,* 2000) but there is no consistent pattern of illness in families that would suggest one simple mode of transmission. In families with affected individuals in two generations, decreasing age of onset of illness between generations (anticipation) has been observed (Horwitz *et al*, 1996; Yuille *et al*, 1998; Goldin *et al*, 1999). However, it is difficult to completely rule out the possibility that the observed anticipation is a result of biased ascertainment.

A number of somatic genetic abnormalities have been described in CLL tumour cells (Dohner *et al,* 2000; Stilgenbauer *et al,* 2000). Using fluorescence *in situ*

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hybridization (FISH) techniques, the most common cytogenetic changes are deletions of 13q14, 11q22–23, 6q21–22 and trisomy 12. Deletions in 17p are seen less frequently and are associated with advanced disease. Mutations in single genes such as *ATM (*11q22–23) and *P53* (17p) have been described in the tumour cells (Stilgenbauer *et al,* 2000). Although studies have found evidence for germ line changes of *ATM* in a small number of sporadic CLL patients (Bevan *et al*, 1999; Bullrich *et al*, 1999; Stankovic *et al*, 1999), this does not seem to account for familial CLL (Bevan *et al*, 1999; Yuille *et al,* 2002). We studied 38 CLL patients in 19 families and did not find any *ATM* mutations although four patients had abnormally low ATM protein levels (Ishibe *et al*, 2003). Human leucocyte antigen (HLA) studies in CLL have found no consistent associations with specific antigens and one linkage study was negative (Bevan *et al,* 2000). Thus, no susceptibility genes have yet been identified that explain the familial aggregation of CLL.

The goal of this study was to conduct a 10-cM genome scan in 18 informative CLL families in order to identify regions of the genome potentially containing a susceptibility gene(s). The regions with cytogenetic abnormalities in tumour cells mentioned above are strong candidate regions for containing germ line genes, but other areas of the genome could be revealed by conducting a complete genome scan.

PATIENTS AND METHODS

Ascertainment of CLL pedigrees. The Genetic Epidemiology Branch of the National Institutes of Health (NIH) has maintained a registry for familial cancers since 1967. Since 1974, families with two or more living cases of CLL have been enrolled, although the majority has been recruited since 1990. This study was approved by an institutional review board (IRB) and informed consent was required and obtained from all subjects in this report. Our practice is to evaluate all affected individuals and first degree relatives of affected patients (above 30 years of age) at the NIH clinical centre or on field trips. In this sample, all CLL patients were diagnosed according to NIH criteria (Cheson *et al*, 1996) based on absolute lymphocyte counts, and examination of peripheral blood and bone marrow, supplemented by flow cytometry. Disease free status was confirmed by medical history, physical examination and complete blood counts. Families have been followed longitudinally, and the diagnostic status of individuals has been regularly updated. The clinical features of 28 CLL kindreds (including 73 CLL patients) have been described in detail elsewhere (Ishibe *et al*, 2001). Eighteen families were judged to be informative for linkage studies, based on the number of available DNA samples from affected and older (> 50 years) unaffected individuals. The remaining families had either incomplete clinical information or DNA samples could not be obtained. For this mapping study, there were a total of 94 individuals with DNA samples, 38 of whom were affected. Two additional patients were not available but their genotypes could be re-constructed based on the genotypes of their spouses and children. Some families consist of affected siblings and others have affected parents and offspring or other multigeneration configurations. Table I shows how the 18 families break down in terms of configuration and number of patients genotyped. In addition, there were 33 unaffected siblings of patients in the sample, with an average age at examination of 61 years. The age at diagnosis in our families was earlier than that in the general population, with a mean of 57·9 years. As in the general population, male patients predominated, with a male:female ratio of 1·6 [this is slightly higher than the 1·35 found in our entire familial sample (Ishibe *et al*, 2001)]. Seventy-five per cent of CLL patients from the families in this linkage study were either Rai stage 0 or 1 at diagnosis. The skew towards earlier stage may reflect the requirement for a family to have two or more living patients in order to be included in our study rather than a genuine clinical difference in stage distribution.

Table I. Number and configuration of affected relatives in 18 CLL families.

Relationship among patients	Number of patients genotyped	Number of families
Siblings	2	8
	3	1
Siblings and cousins	3	1
Parent and offspring	1*	1
	2	3
	3	1
	4	1
Cousin or avuncular	2	2

*No DNA available from the other patients in family.

Genotyping. DNA was extracted from cryopreserved lymphocytes using standard methods. Samples were checked for DNA quality and familial identity using the PROFILERPLUS identity panel [Applied Biosystems (ABI), Foster City, CA, USA]. All samples were screened with 359 markers in 28 panels from the MD-10 Linkage Mapping Set v2 (ABI). Polymerase chain reaction (PCR) products were analysed with either a 3700 or 3100 automated DNA sequencer (ABI). The data were analysed with GENESCAN and GENOTYPER software (ABI). All genotype scoring was done without knowledge of the family pedigree information. To further study a region on chromosome 13, four markers (D13S218, D13S263, D13S156, D13S153) from the original LMSv2 set were repeated with an additional four markers (D13S1296, D13S1306, D13S219, D13S1320) on all the original samples plus an additional (19th) family that had been added to the study.

The average heterozygosity of these markers was 79% and the average interval between markers was 9·5 cM. Sex-averaged Co-operative Human Linkage Center (CHLC)/ABI reference maps were used for the analyses. Genotypic and phenotypic data were stored in DISCOVERY MANAGER 2·3

(Genomica, Boulder, CO, USA) and exported into analysis programs. Families showing non-mendelian transmission of genotypes were examined manually. The individual genotypes that were most likely to be errors were set to missing. The program SIMWALK2 (Sobel & Lange, 1996) was used to identify individual genotypes that had a high probability of being errors, given the marker map distances and order. Genotypes were deleted if they had a probability of 25% or higher of being errors. The total number of genotypes excluded was less than 1% of all the genotypes in the sample, indicating a high level of data quality control. Marker allele frequencies were estimated from the data using the program ILINK from the LINKAGE package (Lathrop & Lalouel, 1984; Lathrop *et al*, 1984; Cottingham *et al*, 1993; Schäffer *et al*, 1994).

Statistical analysis. The power to detect linkage can be estimated under various mode of transmission assumptions. We estimated the power to detect linkage using the program, SLINK (Ott, 1989; Weeks *et al*, 1990), under assumptions of dominant and recessive inheritance (with reduced penetrance) and varying levels of heterogeneity, assuming close linkage (theta = 0·01) to a highly polymorphic marker locus. We generated 200 replicates under each model to calculate the power of detecting limit of detection (lod) scores of 1–3. The models chosen for the simulation are similar to those used in the linkage analyses and are described below.

Multipoint linkage analyses were conducted using both parametric and non-parametric analyses using GENEHUNTER-PLUS (Kruglyak *et al*, 1996; Kong & Cox, 1997). We calculated lod scores under both dominant and recessive inheritance (allowing for heterogeneity) with reduced penetrance and three risk categories dependent on age. Because we did not have any estimates of genetic model parameters from segregation analysis of familial data, we chose values that were consistent with the life-time risk in the population. The life-time risk (Wun *et al*, 1998) for being diagnosed with CLL in the US population using the SEER data is estimated to be 0·37%. This is probably an underestimate as many patients with CLL are asymptomatic and will not be ascertained in registries. We assumed that the allele frequency was 0·0025 under the dominant model and 0·07 under the recessive model. Penetrance was assumed to be 25% for individuals less than 45 years, 50% for those aged 45–70 years and 75% for those over 70 years. In order to allow for phenocopies, the penetrance for the normal genotypes was 0·001. Both of these models predicted a population prevalence of about 0·45%. The exact parameters did not greatly affect the analysis results. Parametric linkage methods have more power to detect linkage than non-parametric methods and are also robust to misspecification of the model (Abreu *et al*, 1999). However, we also applied non-parametric analyses and calculated National Physical Laboratory, UK (NPL) scores (z-scores) using GENEHUNTER-PLUS. The appropriate statistical threshold for a linkage study has been a subject of some debate (Lander & Kruglyak, 1995; Witte *et al*, 1996). When testing the whole genome for linkage, a stringent threshold is needed (lod scores over 3·0) to declare a finding as significant. Lod scores greater than 2·0 are considered suggestive and most studies consider lod scores of 1·0 (*P*-value = 0·02) to indicate regions that should be followed up in further studies.

RESULTS

There were no regions of the genome that showed significant or suggestive linkage according to the criteria given above for whole genome scans. However, six regions of the genome (see Table II) had lod scores greater than 1·0 or NPL scores greater than 1·8 (*P*-value approximately = 0·02). A graphical view of the multipoint results for all of the chromosomes is shown in Fig 1. Curves were drawn for parametric lod scores and for NPL scores; although as described above, the absolute value of the lod and NPL statistics were not comparable in statistical significance.

Interestingly, four out of the six regions noted in Table II were also candidate regions for CLL based on cytogenetic findings in tumour cells. In the initial genome scan, we calculated a lod score of approximately 1·0 on chromosome 13 in between D13S153 and D13S156. This precise region is the one that is most frequently deleted in CLL tumour cells.

Table II. Regions of the genome showing possible linkage to CLL.

Chromosome	Model	Lod or NPL score	*P*-value	Location (cM)	Marker(s)
1	Dom	1·32	0·006	148	D1S868–D1S206
3	Rec	1·51*	0·016	168	D3S1292
	NPL	1·63	0·038	166	D3S1292
	NPL	1·68	0·034	202	D3S1614
6	Dom	1·23	0·008	128	D6S287–D6S262
12	Dom	1·27	0·007	154	D12S86
	NPL	2·81	0·002	154	D12S86
13	NPL	1·78	0·027	46	D13S156
17	NPL	2·78	0·003	0	D17S849

*Lod score assuming heterogeneity.
Dom, dominant; Rec, recessive; NPL, National Physical Laboratory (UK).

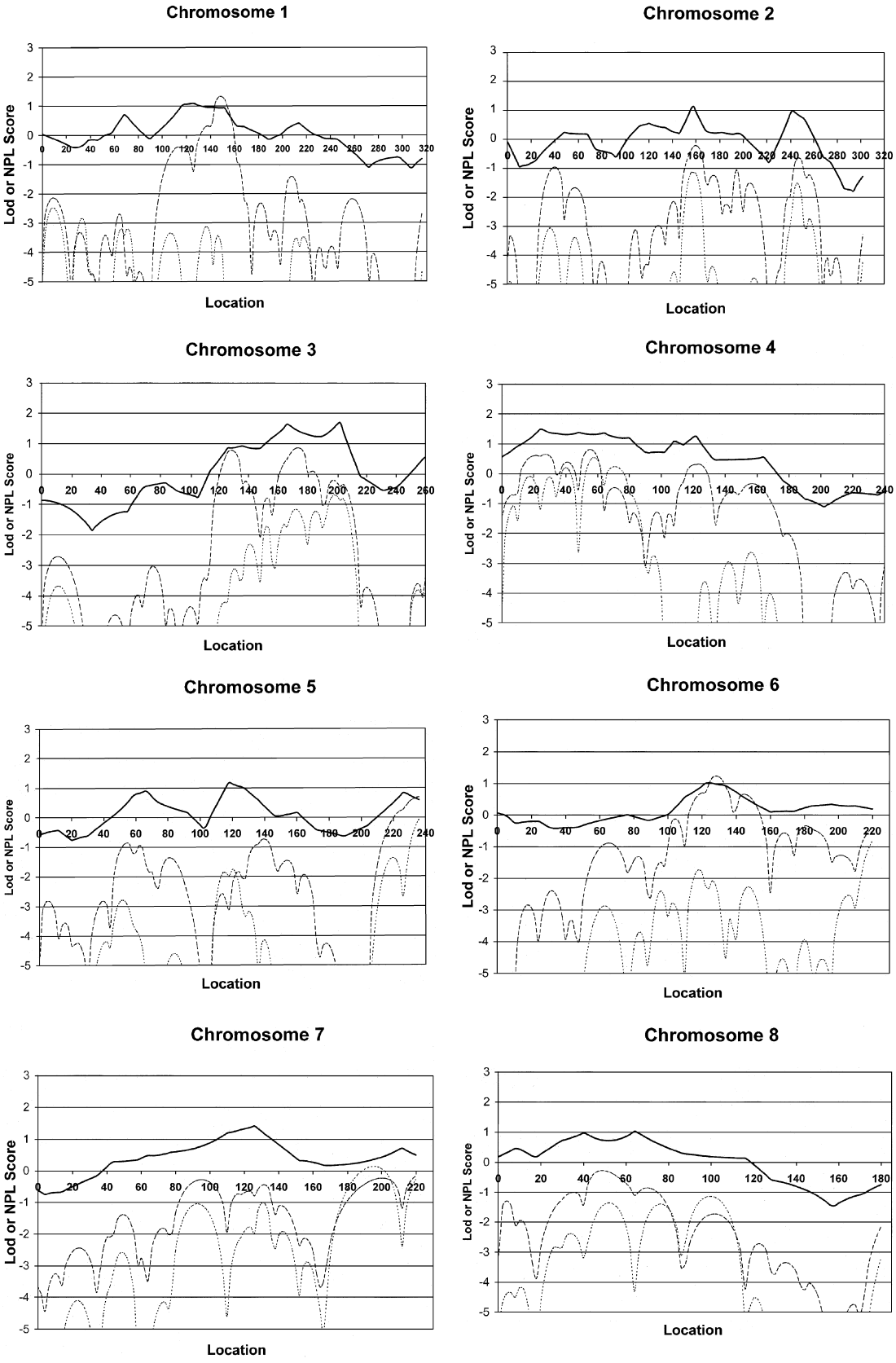

Fig 1. *continued*

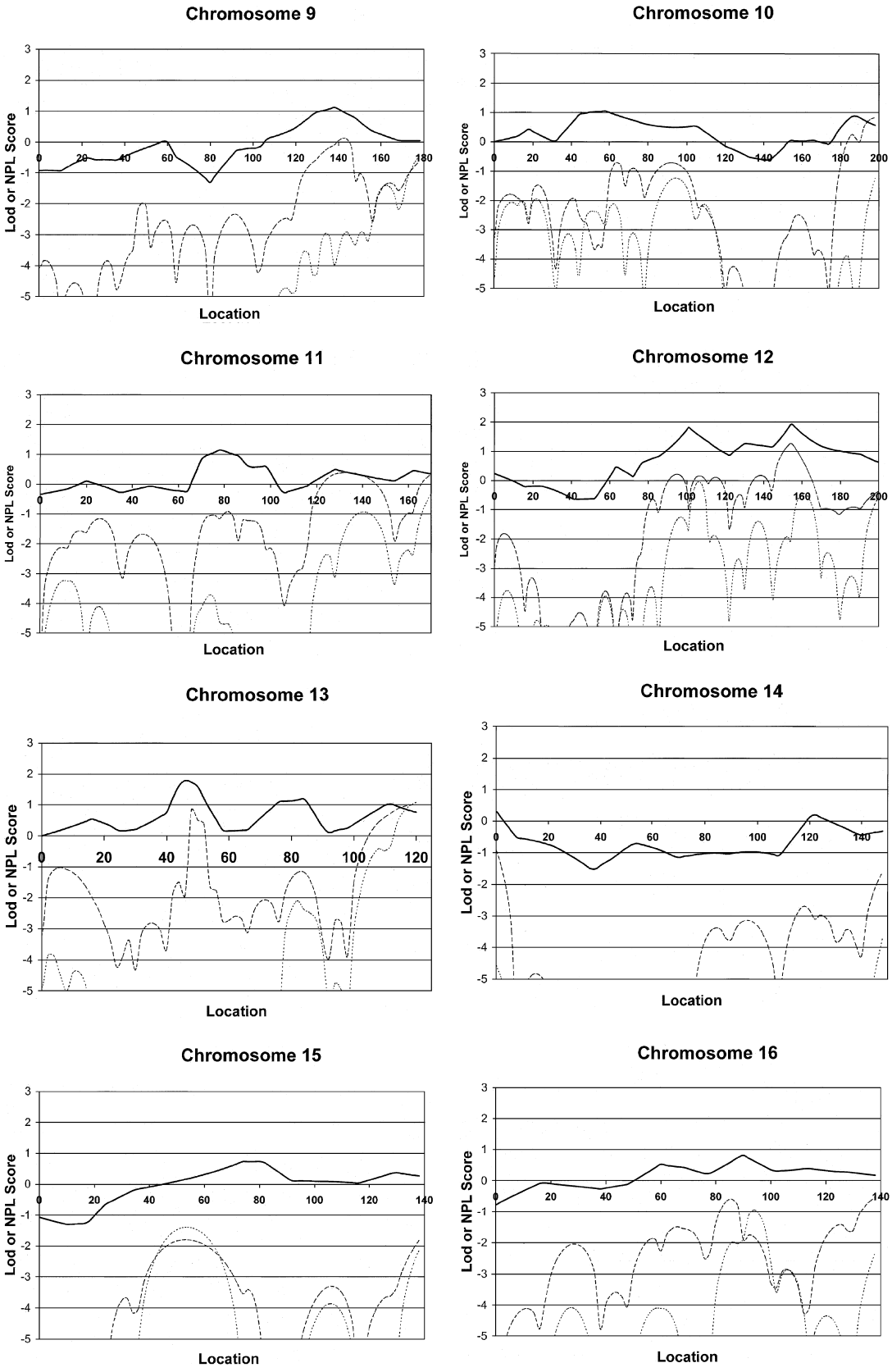

Fig 1. *continued*

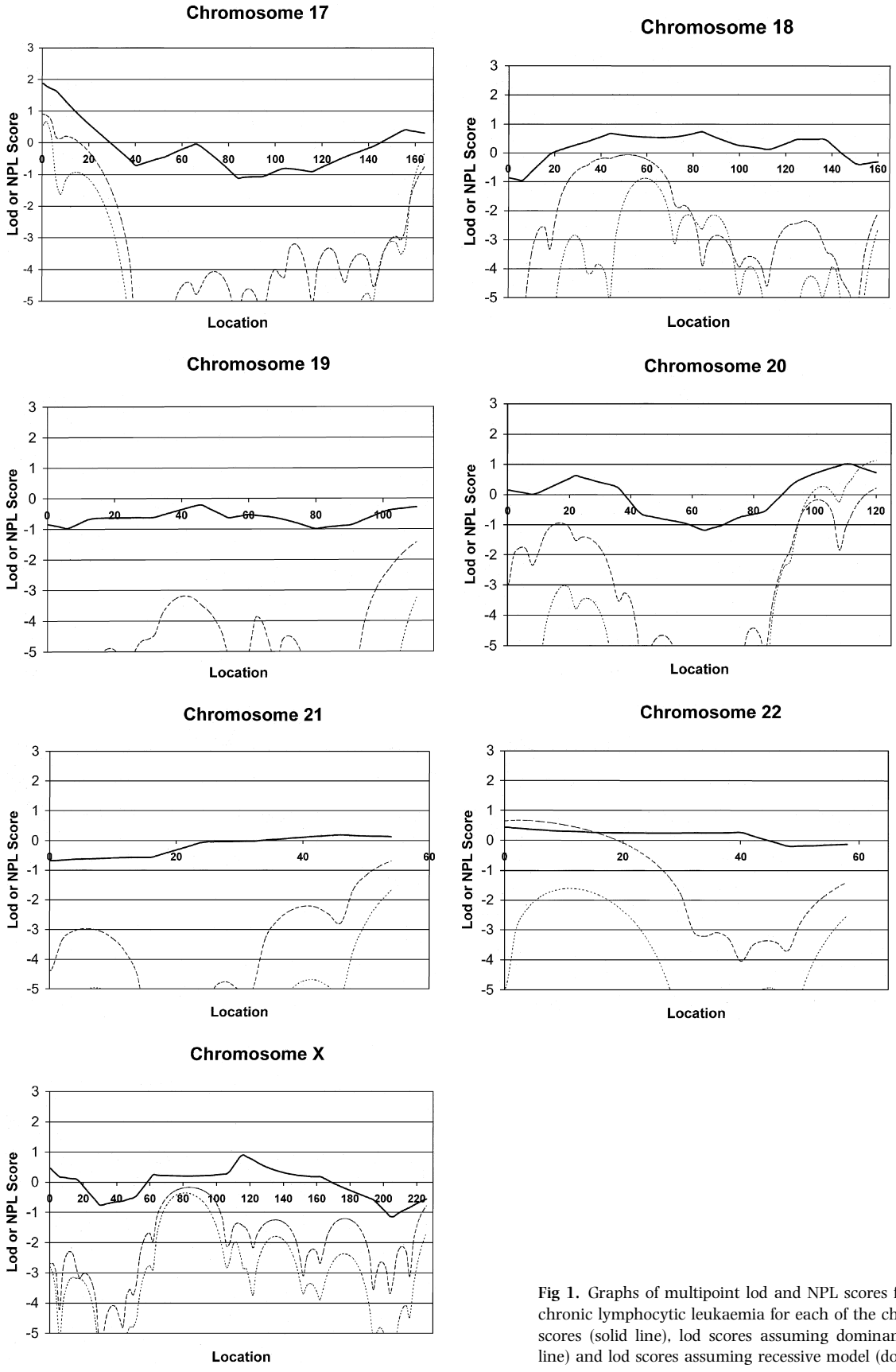

Fig 1. Graphs of multipoint lod and NPL scores for the linkage of chronic lymphocytic leukaemia for each of the chromosomes: NPL scores (solid line), lod scores assuming dominant model (dashed line) and lod scores assuming recessive model (dotted line).

As described in the *Patients and methods*, we attempted to extract more linkage information in this region by genotyping four additional markers from the 5 cM map (D13S1320, D13S1306, D13S1296, D13S219). One additional nuclear family was available for analysis and was genotyped for these markers. When these additional genotypes were considered, the lod score decreased slightly but the NPL score in the region around D13S156 was 1·78 (Table II, *P*-value = 0·03). Chromosome 12 showed positive evidence for linkage under both parametric ($P = 0{\cdot}007$) and non-parametric ($P = 0{\cdot}002$) analyses. Trisomy 12 is a common somatic abnormality in CLL. The critical region of chromosome 12 has not been narrowly identified but there have been partial trisomies observed that narrowed the region to 12q12–q21, which includes the region we identified here. A recently described loss of function mutation in the cytolytic *P2X7* receptor gene was found to be associated with an increased risk for CLL (Wiley *et al*, 2002). This gene is located approximately 3 cM from the microsatellite marker D12S86, which had the peak lod score on this chromosome (Table II). The lod score of 1·3 on chromosome 6 was in the region 6q22–q23, which may overlap with the region deleted in tumour cells. The finding on chromosome 17 overlapped the *P53* gene, which is also a candidate region for CLL. The scores on chromosome 1 and 3 were not in regions with common cytogenetic abnormalities in CLL.

Although these findings are interesting, we note that the power of detecting linkage in our sample is limited. Based on our simulations, if at least 75% of the families were segregating for the same locus, then the power to obtain suggestive evidence for linkage would be high (> 82% if the gene is recessive and > 58% if the locus is dominant). If only half of the families were linked, then we have at least a 50% chance of obtaining a lod score greater than 1·0 ('weak' evidence) but low power to obtain strong evidence for linkage. Power under the non-parametric models would be expected to be somewhat lower (Abreu *et al*, 1999).

DISCUSSION

We did not find any regions of the genome which definitely showed linkage to CLL but the regions identified as 'positive' should be followed up in further studies, especially those that coincided with cytogenetic abnormalities. As candidate genes are identified in these regions, they can be screened for the presence of germ line mutations in CLL patients.

Nineteen CLL patients from 10 families (overlapping with this sample) are currently being screened for cytogenetic abnormalities, using conventional G-bands and FISH. Preliminary results indicate that 14/19 patients show deletions of chromosome 13q14, with two of these 14 also showing trisomy 12 and one patient also showing an 11q deletion. One patient shows only trisomy 12. Abnormalities are not always concordant within families. In a larger study, it might be possible to attempt to correlate cytogenetic findings, V_H mutational status or other sources of clinical heterogeneity with linkage findings. Our sample is limited in power to detect a gene if there is substantial genetic heterogeneity. Further studies using a larger numbers of families are needed to detect genes with greater certainty and to determine the cancer risks associated with these genes.

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